IN03/296

सत्यमेव जयते

INTELLECTUAL PROPERTY INDIA

GOVERNMENT OF INDIA
MINISTRY OF COMMERCE & INDUSTRY,
PATENT OFFICE, DELHI BRANCH,
W - 5, WEST PATEL NAGAR,
NEW DELHI - 110 008.

I, the undersigned, being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application*** *and* ***Complete Specification*** *filed in connection with Application for Patent No.**271/Del/03** dated **12th March 2003.***

Witness my hand this 7th Day of October 2003.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

0271-03

12 MAR 2003

FORM 1

THE PATENTS ACT, 1970
(39 OF 1970)
APPLICATION FOR GRANT OF A PATENT

1. I, (a) the **Director General**

   (b) Defence Research & Development Organisation
   Ministry of Defence, Govt of India,
   B-341, Sena Bhawan, DHQ P.O.
   New Delhi – 110011

   02053

   (c) an Indian National

2. hereby declare -

   (a) that I in possession of of an invention titled :

   AN AGGLUTINATION REAGENT AND A KIT FOR RAPID DETECTION OF TYPHOID

   (b) that the ~~Provisional~~/Complete Specification relating to this invention is filed with this application.

   (c) that there is no lawful ground of objection to the grant of a patent to me.

3. further declare that the inventor(s) for the said invention are :-

   (a) Names (Principal Names first)
   (i) GANGA PRASAD RAI
   (ii) GAURI SHANKAR AGARWAL
   (iii) SHRI KRISHNA SHARMA
   (iv) DEVENDRA KUMAR JAISWAL
   (v) KRISHNAMURTHY SEKHAR
   (vi) KAJAL ARORA
   (vii) VIJAY KUMAR CHAUDHARY

   (b) Address :

   For (i) to (v) - Defence Research & Development Establishment, Jhansi Road, Gwalior - 474 002, INDIA.

   For (vi) and (vii) - Department of Biochemistry
   Delhi University South Campus,
   Benito Juarez Road, New Delhi -110 002,
   INDIA.

DUPLICATE

(c) Nationality : All Indian Nationals.

4. I, claim the priority from the application(s) filed in convention countries, particulars of which are as follows :-

(a) Name of the country : Nil
(b) Application No. : Nil
(c) Date of Application : Nil
(d) Applicant in Convention Country : Nil
(e) Title of Invention in the Convention Country : Nil

5. I state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which I am the applicant/patentee.

(a) Application No. or Patent No. : Nil
(b) Date of Application or Date of Patent : Nil

6. I state that the application is divided out of my application, the particulars of which are given below and pray that this application deemed to have been filed on NA under Section 16 of the Act.

(a) Application No. including Published Sr. No., if any : Nil
(b) Date of filing of Provisional/Complete specifications : Nil

7. That I am the assignee of the true and first inventors.

8. That my address for service in India is as follows : M/S L.S. DAVAR & Co., Patent & Trademarks Attorney, 5/1, (First Floor), Kalkaji Extension, New Delhi - 110 019.

9. Following declaration was given by the inventors :

We the true and first inventors for this invention declare that the applicant herein is our assignee-

(a) Names (Principal Names first)
(i) GANGA PRASAD RAI
(ii) GAURI SHANKAR AGARWAL
(iii) SHRI KRISHNA SHARMA
(iv) DEVENDRA KUMAR JAISWAL
(v) KRISHNAMURTHY SEKHAR
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Delhi University South Campus, Benito Juarez Road, New Delhi - 110 002, INDIA.

(c) Nationality : All Indian Nationals

Signature of Inventor (s) (Full name in brackets)

(i) GANGA PRASAD RAI

(ii) GAURI SHANKAR AGARWAL

(iii) SHRI KRISHNA SHARMA

(iv) DEVENDRA KUMAR JAISWAL

(v) KRISHNAMURTHY SEKHAR

(vi) KAJAL ARORA

(vii) VIJAY KUMAR CHAUDHARY

10. That to the best of my knowledge, information and belief, the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me on this application.

11. Following are the attachment with the application :

(a) ~~Provisional~~/Complete specification
(b) Drawings Nil
(c) Formal Application forms in triplicate
(d) Form-3
(e) Fee of Rs. 5000/-

I request that a patent may be granted to me for the said invention.

Dated this 5th day of March, 03

(AVINASH KUMAR)
DY. DIRECTOR(IPR)
FOR DG, DRDO

To

The Controller of Patent
The Patent Office
New Delhi

0271-03

THE PATENTS ACT, 1970 12 MAR 2003

COMPLETE

SPECIFICATION

SECTION 10

TITLE

AN AGGULUTINATION REAGENT AND A KIT FOR RAPID DETECTION OF TYPHOID

DUPLICATE

APPLICANT

Director General, Defence Research & Development Organisation Ministry of Defence, Government of India, B-341, Sena Bhawan, DHQ P. O., New Delhi-110 011 INDIA, an Indian national

The Following specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed.

FIELD OF INVENTION

The present invention relates to an agglutination reagent and a kit for rapid and early detection of *Salmonella typhi* in serum.

PRIOR ART

Typhoid is an endemic febrile disease caused by *Salmonella typhi*. Typhoid is a major concern of public health. The organism usually enters the body by consumption of contaminated food or water and penetrates the intestinal wall. After that it multiplies and enters blood stream within 24-72 hours resulting in enteric fever and bacteremia. After an incubation period of 10 to 14 days, early symptoms of typhoid, like headache, fever, loss of appetite, bradycardia, splenomegaly etc. appear. Typhoid is diagnosed either by blood culture or by detection of its antigens or by the detection of its antibodies in the blood.

One of the most adapted methods for diagnosing the typhoid fever is the performance of "Widal test", a serological test based on the detection of antibodies in the blood. This test is based on the fact that antibodies against typhoid, remain in the blood of infected person, bind to the bacteria and results in the clumps formation which is referred as "Widal Agglutination".

One of the limitation of the widal test is that the test is not specific as it cross reacts with other febrile organisms and many organisms of family Enterobacteriaceae.

Another limitation of the widal test is that, as typhoid is an endemic disease hence there always exist some background level of antibody in the endemic areas. Hence it becomes necessary to determine the cut-off titre for each region to rule out the possibility of diagnosis as false positive.

Yet another limitation of the widal test is that it gives positive results only after one or two weeks of the onset of fever.

Still another limitation of the widal test is that test it is to be performed on paired serum samples taken at an interval of at least one week apart because single widal test is elusive and inconclusive.

Further limitation of the widal test is that the antibiotic administration in the early phase of infection, inhibits the development of the antibody and hence test may give false negative result.

Still further limitation of the widal test is that TAB vaccinated normal healthy persons give false positive reaction in widal test due to presence of circulating antibody against vaccine in human system.

Another limitation of the widal test is that it gives indirect evidence of typhoid infection.

Further limitation of the widal test is that the test has low sensitivity and low specificity.

Other technique known for diagnosis of typhoid is based upon isolation and identification causative agent. This procedure is termed as golden standard.

In this technique Salmonella typhi is isolated from blood and identified by microscopic and biochemical tests. However, this technique has many limitations.

One limitation of the above technique is that it is time consuming as it requires long period of incubation from 3 days to 14 days and also requires elaborate laboratory facilities.

Another limitation of the above technique is that for its performance large quantity of blood sample (10 ml/patient) is required.

Yet another limitation of the above technique is that it needs large volume of culture medium i.e. 100 ml (10 times of blood sample).

Still another limitation of the above technique is its low sensitivity (40 to 60%), as there are very few organism in circulation, as low as 1/ml which leads to false negative results.

Further limitation of above method is that bacterial growth in culture is inhibited by serum bactericidal agents, present in blood which may lead to false negative results.

Still further limitation of blood culture is that antibiotics treatment during early phase of infection may inhibit bacterial growth in culture which may give false negative results.

Other known techniques such as Radioimmunoassay (RIA), Enzyme - linked immunosorbent assay etc. are based on detection of circulating antigen in the body fluids, but these techniques have many limitations.

One limitation of these techniques is that they require sophisticated and elaborate laboratory facilities.

Another limitation of RIA is that it requires radioactive material which is health hazard and also needs trained personnel to handle the radioactive material.

Still further limitation of above techniques is that reagents are expensive.

Further limitation of these techniques is that minimum 4-5 hours are required to perform the tests.

OBJECT OT THE INVENTION

The primary object of the present invention is to provide an agglutination reagent for rapid and early detection of S.typhi is serum samples of suspected typhoid patients.

Another object of the present invention is to provide an agglutination reagent which enables diagnosis of typhoid within 3 minutes after collection of serum samples.

Yet another object of the present invention is to provide an agglutination reagent which requires serum sample as small as 20µl for diagnosis of typhoid disease.

Further object of the present invention is to provide an agglutination reagent which enables detection of typhoid bacteria by a simple latex agglutination technique.

Still further object of the present invention is to provide an agglutination reagent which enables specific identification of Salmonella typhi antigen in serum samples of suspected typhoid patients.

Yet further object of the present invention is to provide an agglutination reagent which enables the diagnosis of typhoid in the early stages of infection, even within one or two days after the onset of the fever.

Even further object of the present invention is to provide an agglutination reagent which is highly sensitive.

Yet further object of the present invention is to provide an agglutination reagent which enables diagnosis of the disease in field conditions as it does not require any equipment of laboratory facility.

Still further object of the present invention is to provide an agglutination reagent which does not require any specially trained personnel to perform the test.

Even further object of the present invention is to provide an agglutination reagent kit which enables the diagnosis of typhoid even in those patients who have been administered with antibiotics resulting in blood culture isolation as negative.

DESCRIPTION OF INVENTION

According to this invention there is provided an agglutination reagent for rapid and early detection of typhoid comprising 1% carboxylated latex particles in a buffer-ase coated with Salmonella typhi specific antibody suspended in storage buffer. The present invention proposes an agglutination reagent for detection of typhoid in serum of suspected typhoid patients. The process for the preparation of the agglutination reagent has been described in the Co-pending Indian patent application no. 1187/DEL/2002. According to the preferred embodiment of the present invention, the agglutination reagent is prepared by a process comprising of following steps:

(a) **Preparation of antibody (immunoglobulins):**

Flagellin gene sequence specific to Salmonella typhi is cloned and expressed by recombinant DNA technology. The expressed recombinant protein is purified by affinity chromatography. Hyper immune sera against this recombinant protein is raised in rabbit. Immunoglobulin fraction of hyper immune sera is separated by ammonium sulphate precipitation. The precipitated immunoglobulins are suspended in 50mM phosphate buffer (pH7.2), dialysed and protein content determined.

(b) **Preparation of Latex Particles suspension:**

1% carboxylated latex particles of size 0.88 to 0.90 μm and 40mM 2-N Morphilinoethane sulphonic acid (MES) buffer (pH 5.5-6.0) are taken in a preferred ratio of 1:1 in a tube. They are mixed on a vortex mixer for around 60 seconds and centrifuged at 10,000 rpm for 10-12 minutes at about 4°C. The latex particles are further washed twice in 20mM MES buffer of pH 5.5 by mixing on vortex mixer for around 60 seconds, followed by centrifugation at 10,000 rpm for 10-12 minutes at about 4°C.

After the final wash, the latex particles are suspended in 20mM MES buffer of pH5.5 and the volume is made up equal to the starting volume of the latex particles. The suspension is then sonicated by a tip sonicator at about 5 watts for 60-120 seconds, preferably 90 seconds. To this suspension freshly prepared solution of 0.1 M 1-ethyl-3(3-dimethyl-amino-propyl) carbodimide hydrochloride (EDC) in 20mM MES buffer (pH 5.5) taken in the preferred ration of 1:1, is added drop wise , while the solution is slowly vortexed. The tube is rotated slowly end-over-end for about 3 hours at a temperature of 20-25°C. It is then washed thrice with 20mM MES buffer (pH 5.5) at 10,000 rpm for 10-12 minutes at a temperature of about 4°C. The latex particles are resuspended in MES buffer (20mM, pH5.5) and sonicated for 60-120 seconds by a tip sonicator at 5 watts.

(c) Coating of Latex Particles with Antibody (immunoglobulins):

To the suspension of latex particles prepared in step(b), 0.6-1.0 mg preferably 0.8 mg per ml of the suspension, immunolobulins prepared in step (a) are added. The whole mixture is then rotated end-over-end for 18-20 hours at a temperature of 20-25°C. The coating reaction is stopped by addition of 1M glycine (pH 11.0) taken in quantity of 0.06ml per ml of solution of immunoglobulin coated latex particles. Rotation is continued for about 30 minutes at a temperature of 20-25°C. The coated latex particles are pelleted out by centrifugation at 10,000 rpm for 10-12 minutes at a temperature of about 4°C. The pellet is washed thrice with washing buffer (50mM glycine, pH8.5; 0.03% triton X-100 and 0.05% sodium azide) at 10,000 rpm for 10-12 minutes at a temperature of about 4°C. Finally the washed coated latex particles are resuspended in storage buffer (50mM glycine, pH8.5; 1.0% bovine serum albumin; 0.03% triton X-100; 0.1% sodium azide and 0.01% thiomersol) to a final concentration of 1% and sonicated by a tip sonicator for around 60 seconds at about 5 watts and stored at 4°C.

The kit for rapid detection of typhoid is made comprising of – 1% agglutination reagent, glass slides, droppers, wooden sticks and positive & negative controls.

METHOD OF USE

(a) Take 20-40µl (1 to 2 drops) of test serum, positive and negative controls at three distinct places on a glass slide

(b) Add 10-2µl (1-2 small drops) of latex reagent to test serum, positive and negative controls

(c) Mix the reactants with separate wooden sticks carefully to avoid any intermixing of reactant placed at separate places and rotate the slide for 1-2 minutes.

A positive reaction is indicated by the development of an agglutination within 1-2 minutes of mixing the reagent with the test sample and positive control, showing clearly visible clumping of the particles. The speed of appearance and quality of agglutination depends on the strength of the antigen present, varying from large clumps which appear within a few seconds of mixing, to small clumps which develop rather

slowly. In negative reaction the reagent does not agglutinate and the cloudiness or the turbid nature remains substantially unchanged throughout the test.

Laboratory studies on the reliability of proposed agglutination reagent for rapid detection of *Salmonella typhi* in typhoid patient serum is performed with the laboratory strains of Salmonella typhi; and culture proven and widal positive serum samples collected from suspected cases of typhoid; and with serum samples of apparently normal healthy individuals. The result indicate 93.00% sensitivity and 98.00% specificity.

The present invention will now be illustrated with a working example which is intended to be illustrative example and is not intended to be taken restrictively to imply any limitation on the scope of the present invention.

EXAMPLE

Flagellin gene sequence specific to *Salmonella typhi* was amplified by polymerase chain reaction (PCR) using gene specific primers. Amplified PCR product was cloned in Glutathione-S-transferase (GST) vector and later expressed. The expressed protein was purified by GST affinity column chromatography. The protein content of the purified product was determined by Bradford method. Hyper immune serum against this protein was raised in rabbit. Immunoglobulins fraction of hyper immune sera was separated by ammonium sulphate precipitation. The precipitated immunoglobulins were suspended in 1.0 ml PB (50 mM, pH 7.2), dialysed and protein content determined. 1.0 ml of 1% carboxylated latex particles and 1.0 ml of 40 mM MES buffer (pH 5.5 - 6.5) were taken in 2.0 ml microcentrifuge tube. Then they were mixed on vortex mixer for 60 seconds and centrifuged at 10,000 RPM for 10-12 minutes at a temperature of 4°C. The latex particles were further washed twice in 2.0 ml of 20mM MES buffer (pH 5.5) by mixing on vortex mixer for 60 seconds and centrifugation at 10,000 RPM for 10-12 minutes at a temperature of 4°C. Following the final wash, the latex particles were suspended in 1.0 ml MES buffer (20mM, pH5.5) and sonicated by a tip sonicator at 5 watts for 60-120 seconds. Later 1.0 ml of freshly prepared solution of 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC) in MES buffer (20mM, pH5.5) was added drop wise while the solution was slowly vortexed . Then the tube was rotated slowly end-over-end for 3 hours at a temperature of 20-25°C followed by washing three times with MES buffer (20mM, pH 5.5) at 10,000 RPM for 10-12 minutes at a temperature of 4°C. The latex particles were resuspended in 0.7 ml MES buffer (20 mM, pH 5.5) and sonicated for 60-120 seconds by a tip sonicator at 5 watts. 0.8 mg of immunoglobulins were added to latex particles and volume was made up to 1.0 ml with MES buffer (20mM, pH 5.5). This was then rotated end-over-end for 18-20 hours at a temperature of 20-25°C. The coating reaction was then stopped by addition of 0.06 ml of 1M glycine (pH 11.0). The rotation was continued for 30 minutes at a temperature of 20-25°C. The coated latex particles were pelleted out by centrifugation at 10,000 RPM for 10-12 minutes at a temperature of 4°C. The pellet was washed thrice with 2.0 ml of washing buffer (50 mM glycine, pH 8.5, 0.03% triton X-100 and 0.05% sodium azide) at 10,000 RPM for

10-12 minutes at a temperature of 4°C. The washed coated latex particles were resuspended in storage buffer (50mM glycine, pH 8.5, 1.0% bovine serum albumin, 0.03% triton X-100, 0.1% sodium azide and 0.01% thiomersol) to a final concentration of 1% and sonicated with tip sonicator for 60 seconds at 5 watts and stored at 4°C.

It is to be understood that the present invention is susceptible to modifications, changes and adaptations by those skilled in the art. Such modifications, changes, adaptations are intended to be within the scope of the present invention which is further set forth under the following claims:-

WE CLAIM:

1. An agglutination reagent for rapid and early detection of typhoid comprising 1% carboxylated latex particles in a buffer-ase coated with Salmonella typhi specific antibody suspended in storage buffer.

2. An agglutination reagent as claimed in claim 1 wherein the size of latex particles is preferably 0.88 to 0.90 μm.

3. An agglutination reagent as claimed in claim 1 wherein buffer for latex particles is preferably 2-N Morphilinoethane sulphonic acid (MES)

4. An agglutination reagent as claimed in claim 1 wherein the pH of the buffer for latex particles is preferably 5.5 to 6.0.

5. An agglutination reagent as claimed in claim 1 wherein the ratio of latex particles and buffer is preferably 1:1.

6. An agglutination reagent as claimed in claim 1 wherein the storage buffer comprises 50MM glycine of pH 8.5, 1% bovine serum albumin, 0.03% triton X-100, 0.1% sodium azide and 0.01% thiomersol.

7. An agglutination reagent substantially as herein described and illustrated.

Dated this 5th day of March 2003

(G.S.DAVAR)
OF L.S.DAVAR & CO.,
APPLICANTS' ATTORNEY

ABSTRACT OF INVENTION

According to the present invention there is provided an agglutination reagent which enables detection of typhoid in serum of suspected typhoid patient within 3 minutes of collection of serum samples. The disease can be diagnosed during early stage of infection, and also in the patients who are under antibiotics treatment. According to the present invention Salmonella typhi specific antibody is prepared by recombinant DNA technology using flagellin protein specific to S.typhi and then raising the hyper immune sera to this recombinant protein, in rabbits. The immunoglobulin fraction of the hyper immune sera is separated by ammonium sulphate precipitation. The precipitated immunoglobulins are suspended in phosphate buffer and dialysed. This antibody is coated on washed and sonicated latex particles. The latex particles coated with Salmonella typhi specific antibody are suspended in storage buffer to a final concentration of 1% and stored at 4°C. Though the agglutination reagent of the present invention is stored at 4°C but is stable even at 20-25°C for months. The method of testing is simple and can be performed in field conditions without the use of any laboratory facilities and instruments and also without any specialized training. The serum sample required for test by the method of present invention is only 20-40µl as compared to the conventional techniques which require a sample of 10ml or more of blood and the result is obtained in hours and many times in days.